HIGHLIGHTED TOPIC | Physiological Imaging of the Lung

Real-time lung microscopy

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Kuebler WM, Parthasarathi K, Lindert J, Bhattacharya J. Real-time lung microscopy. J Appl Physiol 102: 1255–1264, 2007. doi:10.1152/japplphysiol.00786.2006.—Although proinflammatory cell signaling in the alveolocapillary region predisposes to acute lung injury, key cell-signaling mechanisms remain inadequately understood. Alveolo-capillary inflammation is likely to involve coordinated signaling among cells of different phenotypes. For example, migration of inflammatory cells into the alveolus might entail coordinated signaling between adjoining alveolar epithelial and microvascular endothelial cells. The popular cultured cell experimental strategy fails to replicate this multicellular environment. Cultured lung cells, both alveolar and endothelial, undergo phenotypic transformations; hence they might inadequately reflect innate responses of native cells. Consequently, new approaches are required for the investigation of cell signaling in the native setting. Here we summarize new developments in classical intravital microscopy and discuss real-time fluorescence imaging as a novel technique for studying second-messenger mechanisms in the alveolo-capillary region.

calcium ion; mitochondria; mitochondrial calcium ion; reactive oxygen species; nitric oxide; P-selectin; surfactant

INTRAVITAL MICROSCOPY

Intravital microscopy (IVM) entails video microscopy of the bright-field illuminated lung surface. The alveolocapillary region accessible to IVM consists of alveoli and associated juxta-alveolar arteriolar, septal, and venular microvessels. Divergent vs. convergent flows distinguish arteriolar from venular microvessels, respectively. In the rodent lung, the microvessels are anatomic “capillaries,” since none exceed ~30 μm in diameter and none contain smooth muscle (80). Recent advances in IVM are attributable to window preparations for direct visualization of subpleural lung microvessels and alveoli in the intact thorax. Early preparations were established by Wearn and coworkers (85) and Terry (78). More recently, lung IVM was approached in dog by Wagner (81) and in rabbit by Kuhnle et al. (42) by implanting flanged steel windows in the thoracic wall. The implants stabilize the lung surface by means of suction applied through a system of boreholes, thereby enabling IVM in the presence of lung ventilation.

Window approaches have enabled quantitative analyses in lung microvasculature in vivo. Visualization of blood flow by fluorescent plasma markers or erythrocytes (Fig. 1A) has facilitated direct measurement of lung capillary transit times, which were found to be in the range of 12.7 ± 3.2 s and thus markedly exceeded prior estimates (83). Subsequent studies identified the dynamic regulation of capillary recruitment and derecruitment by changes in cardiac output, alveolar oxygen tension, and local rheological factors (41, 57, 82). Fluorescence labeling of circulating white blood cells (Fig. 1B) yielded important information on the size, site, and regulation of the marginated pool of leukocytes and helped to characterize kinetics and mechanisms of leukocyte sequestration in lung inflammation. Studies by Lien et al. (45) and Kuebler et al. (36) identified the alveolar capillary bed as the predominant site of physiological leukocyte margination in the lung. During their passage through the alveolar capillary network, >90% of leukocytes become retained at least once for varying periods of time, ranging from <1 s up to >20 min (34, 45). Leukocyte retention results in the accumulation of a marginated pool, which equals the total number of circulating leukocytes in blood (33). Whereas physical deformation of leukocytes from a spherical into an elongated shape suggests mechanical hindrance of leukocyte passage through lung capillaries as the predominant mechanism underlying leukocyte margination (19), marked acceleration of leukocyte transit through lung capillaries by a nonspecific selectin inhibitor, fucoidin, suggests an additional contribution of adhesion molecule-mediated cell/cell interaction (35).

The availability of transgenic mouse or rat models has further propelled lung IVM in vivo (48, 61, 64). Recently, Tabuchi et al. (77) reported a new IVM model in mice that provides excellent images of subpleural pulmonary arterioles, venules, and capillary networks (Fig. 1C) and of dynamic cell-cell interactions in pulmonary microvessels (Fig. 1D). In this model, the predominant site of hypoxic pulmonary vasocstriction could be localized in small arterioles >30 μm (77). The possible use of transgenic animals yields additional

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potential for the analysis of intra- and intercellular signaling pathways in these models. Transgenic expression of biofluorescent proteins, such as GFP under cell-specific promoters, furthermore facilitates the study of specific cell subsets and circumvents problems inherent to synthetic fluorophores (74).

Two notable IVM models have been proposed for chronic studies of inflammatory processes in the lung. Fingar and coworkers (18) implanted a lung chamber into the right thoracic wall of anesthetized rats. The chamber allows for microscopic investigation of the lung surface after the animals resume spontaneous respiration and showed progressive vascular leakage of fluorescent dyes from pulmonary capillaries into lung alveoli in oleic acid-induced lung injury. However, long-term observations in this model are yet lacking. Sikora and coworkers (70) chose a different approach by transplanting lung tissue into the dorsal skinfold chamber of nude mice (44). After revascularization, these lung allografts maintain characteristics of pulmonary vasoreactivity in that they undergo hypoxic vasoconstriction (71). Nicotine was shown to induce leukocyte rolling and adhesion via activation of MAPK and P-selectin in this model, suggesting that these mechanisms may be relevant in nicotine-induced airway inflammation (71).

In vivo preparations for IVM of leukocyte trafficking in the lung bear the advantage of closely reflecting complex hemodynamic, metabolic, and inflammatory conditions in the intact organism. Yet they pose manifold technical challenges and limitations, which arise from the need to create optical access to the lung, to minimize motion artifacts due to respiratory and cardiac movements, and to control global and local hemodynamics, which directly affect the kinetics of cell trafficking (34, 41).

Isolated lung preparations circumvent difficulties innate to intact preparations. Blocking antibodies and indirect immunofluorescence imaging of endothelial adhesion molecules have been used in the isolated lung to identify the role of adhesion molecules in leukocyte sequestration. These studies suggest that, in hypoxic, bleomycin-, or ventilator-induced lung injury, ICAM-1 universally mediates leukocyte entrapment in lung capillaries and rolling in pulmonary venules (53, 56, 68). In contrast, selectins seem to contribute differentially to these effects in that P-selectin mediates leukocyte margination in response to mechanical stimuli, such as hydrostatic stress (25) or overventilation (53), whereas L-selectin regulates the response to hyperoxia (56).

Nevertheless, some difficulties are that the isolated, perfused lung preparation may, per se, alter leukocyte kinetics because of cell activation by artificial surfaces and the need for anticoagulation (5). Moreover, leukocyte labeling, both in and ex vivo, carries problems. Ex vivo separation and labeling of cells allows for the study of specific leukocyte subsets, e.g., neutrophils or monocytes at the cost of possible cell activation. In vivo labeling by systemic infusion of nuclear or mitochondrial dyes, such as acridine orange or rhodamine 6G, avoids cell separation steps, but does not permit differentiation between different leukocyte subcategories and still cannot exclude dye-dependent alterations in cell trafficking (1, 21). Of note, it has proven difficult to track leukocyte transmigration into the lung interstitial and alveolar space. In vitro, the combination of phase contrast and fluorescence imaging yields important insights into cellular mechanisms regulating leukocyte transmigration (14, 76). In transilluminated preparations, transendothelial and interstitial migration of leukocytes can be clearly visualized and is not attenuated by fluorescent dyes such as rhodamine 6G (50). Yet in epi-illumination preparations, these techniques have proven difficult, potentially due to limitations by two-dimensional imaging techniques. As discussed later, recent advances in optical-sectioning microscopy yield the potential for subsequent three-dimensional image reconstruction and may thus provide a novel approach for the visualization of cell migration kinetics in three-dimensional tissue.

IVM has also been applied to micropuncture studies of lungs in vivo or in isolated, perfused lung preparations. Micropuncture measurements by the servo-null technique alone permit direct determinations of vascular and interstitial pressures in the lung. Micropuncture of sequential lung microvascular segments identified the alveolar septal capillary network as the major site of the total vascular pressure drop across the lung from pulmonary artery to left atrium (8, 22, 55, 69). Pulmonary vasoconstrictors, in contrast, tend to localize to either arterioles or venules as the predominant constriction site, with hypoxia or platelet-activating factor acting primarily in arteriolar segments (54, 63), while mediators such as histamine, endothelin-1, or thromboxane A2 constrict predominantly the venular compartment (62, 63). Micropuncture pressure measurements obtained in the interstitium surrounding alveolar septal junctions and in the adventitial space of 30- to 50-μm-diameter vessels revealed an interstitial pressure gradient that drives perimicrovascular fluid flux from the alveoli to the lung hilum (7, 20). Lung
expansion abolishes this pressure gradient, suggesting that respiratory lung volume changes may cyclically vary interstitial liquid flow and thus generate a pumplike mechanism to facilitate interstitial fluid clearance (6, 43).

**REAL-TIME FLUORESCENCE IMAGING**

Real-time fluorescence imaging (RFI) methods, involving microscopic imaging of the isolated, perfused lung, have enabled the investigation of lung cellular and molecular responses in situ. The imaged field thereby provides an opportunity for obtaining optical data specifically from intact epithelial and endothelial cells of alveoli and microvessels, respectively.

Ca

Lung RFI enables determinations of cytosolic Ca

levels in individual endothelial cells in intact capillaries. This approach led to the recognition of so-called “pacemaker” endothelial cells that are located at some vascular branch points. These branch-point endothelial cells regulate Ca

levels at levels higher than midsegmental cells (Fig. 2A), and they generate intercellular Ca

waves (87). In these in situ endothelial cells, increase of the microvascular pressure markedly increases the amplitude of Ca

cyt oscillations, while causing relatively small or no increases in the mean Ca

levels. These findings reveal that a characteristic response of branch-point endothelial cells to proinflammatory stimuli is to increase Ca

cyt oscillations.

Mechanistic understanding of branch-point Ca

oscillations was approached through Ca

determinations in the endoplasmic reticulum and mitochondria. Ca

uptake and release by the endoplasmic reticulum entails, respectively, endoplasmic reticulum Ca

-ATPase pumps and inositol trisphosphate receptors. Mitochondrial Ca

(Ca

mit) is regulated by Ca

entry through a Ca

-sensitive uniporter (30) and by Ca

extrusion across a Na

/Ca

exchanger. Ca

levels in these organelles were determined by applying the dyes, fura 2-FF and rhod 2.

Fura 2-FF, which has low Ca
-binding affinity, elicits Ca
-sensitive fluorescence in the high-Ca
-environment of the endoplasmic reticulum. In confirmation, Parthasarathi et al. (59) showed that thapsigargin, 2,5-di-(t-butyl)-1,4-hydroquinone, which depletes endoplasmic Ca
-levels, decreases fura 2-FF fluorescence of lung capillaries. The fluorescence of cationic rhod 2, which accumulates specifically in the negatively charged mitochondrial matrix, reports Ca
mit levels. Through membrane permeabilization studies, Ichimura et al. (27) confirmed the mitochondria-specific distribution of rhod 2 in lung endothelium. These studies indicate that fura 2-FF, rhod 2, as well as the mitochondria-labeling dye Mitotracker Green, are most fluorescent at capillary branch points (Fig. 2B), attesting to high-density colocalization of the endo-

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**Fig. 2.** Real-time fluorescence imaging of lung capillaries. A: the image shows furo 4 fluorescence in septal (SEP) and venular (VEN) capillaries surrounding alveoli (ALV). Note high fluorescence denoted by green pseudocolor occurs at the indicated branch point (white arrow). Direction of blood flow is indicated (black arrow). B: image obtained by confocal microscopy shows branch point (BR) region at the junction of a septal and a venular capillary. Note, fluorescence of mitochondrial dye, Mitotracker Green, is dominant in branch point cells (white arrows). An adherent leukocyte (*) shows uptake of fluorescence. C: concentration-dependent diaminofluorescein (DAF) fluorescence response in lung capillaries to infusion of nitric oxide donor S-nitroso-N-acetyl-penicillamine (SNAP). Fluorescence intensity (F) is expressed relative to its individual baseline (F0). D: nitric oxide response to increase of left atrial pressure (PLA). Vessel margins are depicted by line sketch, and direction of blood flow by arrow. Note response is dominant at branch points.
plasmic reticulum with mitochondria in branch-point endothelial cells.

TNF-α decreased fura 2-FF fluorescence, an effect that was blocked by the inositol trisphosphate receptor blocker, xestospongin C, indicating that TNF-α-induced Ca^{2+} cyt oscillations occurred as a consequence of Ca^{2+} release from the endoplasmic reticulum. Importantly, both TNF-α and capillary pressure elevation increased Ca^{2+} mit. These Ca^{2+} mit responses were also xestospongin C inhibitable, indicating that, in branch-point endothelial cells, Ca^{2+} mit uptake occurs secondary to Ca^{2+} release from the endoplasmic reticulum.

Reactive Oxygen Species

Lung RFI has revealed new understanding of mechanisms underlying endothelial reactive oxygen species (ROS) production in lung capillaries. The dyes, dichlorofluorescein (DCF) and hydroethidium, detect capillary fluorescence of different ROS species, namely H_2O_2 and superoxide, respectively (2, 27, 59). Since DCF fluorescence might be attributable to nonspecific factors, such as the excitation light, DCF responses must be interpreted against appropriate controls. By slowing down the imaging acquisition rate, Parthasarathi et al. (59) and Ichimura et al. (27) obtained stable baselines of DCF fluorescence lasting more than 20 min, indicating that progressive light- and self-induced effects (10, 65) were absent. Using this imaging protocol, they showed that H_2O_2 caused dose-dependent increases in DCF fluorescence and that polyethylene glycol-catalase blocked DCF responses, while nitric oxide (NO) inhibitors were without effect. Inclusion of these controls provided a measure of security in interpreting the DCF data.

Al-Mehdi et al. (2) showed that nonhypoxic ischemia increases ROS production. Findings by Brueckl et al. (9) and Zhang et al. (88) indicate that hyperoxia and ischemia also increase lung capillary ROS production. In the studies by Brueckl et al. (9), hyperoxia increased ROS in two successive phases that were, respectively, mitochondria and NADPH oxidase dependent. The studies by Minamiya and coworkers revealed direct evidence for the neutrophil respiratory burst resulting from endotoxin (52) and apoptosis and ROS production in lung endothelial and epithelial cells in a model of reexpansion injury (67). Findings by Parthasarathi et al. (59) and Ichimura et al. (27) indicate that branch-point endothelial cells generate H_2O_2 entirely by mitochondrial mechanisms and that mitochondrial H_2O_2 increases in response to the TNF-α and pressure-induced Ca^{2+} mit increases. These findings indicate that the physiological significance of augmented Ca^{2+} cyt oscillations is to increase Ca^{2+} mit that, in turn, increases mitochondrial ROS production.

In Situ Immunofluorescence

Detection of signaling proteins by in situ immunofluorescence has been applied in conjunction with RFI determinations. In lung microvessels, immunofluorescence revealed expression of the leukocyte adhesion receptor, P-selectin (34), and NADPH-oxidase activation by the small GTPase, Rac1 (9). In alveoli, the approach was used to distinguish type 1 from type 2 cells (25) and to detect activation of the cytosolic phospholipase A_2 (37).

These approaches have revealed the mitochondrial role in proinflammatory signaling. Endothelial P-selectin is held in apical vesicles called Weibel-Palade bodies (WPB). WPB exocytosis causes P-selectin expression on the endothelial surface, thereby marking proinflammatory endothelial activation. Although increase in Ca^{2+} cyt levels is thought to stimulate WPB exocytosis, Parthasarathi et al. (59) and Ichimura et al. (27) showed that, in lung capillaries, H_2O_2 is the critical exocytosis stimulus, since blocking H_2O_2 with polyethylene glycol-catalase blocked P-selectin expression but not Ca^{2+} cyt oscillations.

Taking together these findings from branch-point endothelial cells, it appears that the major function of Ca^{2+} cyt oscillations is to modulate Ca^{2+} mit. Increases in Ca^{2+} cyt oscillations increase Ca^{2+} mit, thereby increasing mitochondrial H_2O_2 production. Mitochondrial H_2O_2 diffuses to cytosolic targets, namely WPB, to cause P-selectin exocytosis and, subsequently, leukocyte adherence (25). Hence endothelial Ca^{2+} cyt oscillations and mitochondria are revealed as critical regulators of proinflammatory signaling in lung capillaries.

Alveolo-capillary Cross Talk

RFI has led to major new understanding of alveolar-capillary signaling. Airway instillation of bacteria, such as P. aeruginosa, induces alveolar leukocyte migration in ~4 h (72). However, relevant signaling mechanisms are inadequately understood. Since epithelial and endothelial barriers restrict and possibly inhibit direct diffusion of chemokines, the chemotactic signal has to be vectorially transmitted from alveoli to capillaries by cross-compartmental signaling.

Addressing this question, Kuebler et al. (37) noted that alveolar microinjection of TNF-α increases Ca^{2+} cyt in both the alveolar epithelium and the adjacent capillary endothelium, revealing the presence of alveolar-capillary signal transmission. The endothelial response occurred in ~5 min, indicating that signal transmission initiates rapidly. Intra-alveolar injection of an antibody against TNF-α receptor-1 blocked the capillary Ca^{2+} cyt response, while intracapillary antibody injection had no effect. These findings rule out alveolar TNF-α leakage as an experimental artifact. Intraalveolar TNF-α injection failed to increase alveolar epithelial Ca^{2+} cyt, pointing to the vectorial specificity of the TNF-α-induced alveolocapillary signal transmission.

Characteristically, the alveolar epithelial response consists of high-amplitude Ca^{2+} oscillations that are absent in the capillary endothelial response, attesting to different patterns of Ca^{2+} mobilization in the two cell types. The oscillations reflect endoplasmic Ca^{2+} release, while the damped endothelial response indicates the presence of direct Ca^{2+} entry as induced by arachidonate (51). Accordingly, alveolar pretreatment with cytosolic phospholipase A_2 inhibitors blocks the TNF-α-induced alveolar-capillary Ca^{2+} transmission (37). These findings indicate that epithelial and endothelial cells of the alveolo-capillary membranes effectively coordinate vectorial transmission of proinflammatory signals.

NO

To allow for direct detection of the diatomic free-radical NO in living cells and tissue, Kojima and coworkers (31) designed and synthesized a new group of fluorescent indicators, the diaminofluoresceins (DAFs). Due to their reactive aromatic vicinal diamines, the weakly fluorescent DAFs are rapidly
developed derivative 4-amino-5-methylamino-2 for DAF-2 and as low as 3 nmol/l in the more recently
ative of the fluorescence response over time. The detection
directly given by fluorescence intensity, but by the first deriv-
dative of the fluorescence response over time. The detection
limit of the assay has been estimated at 5 nmol/l in living cells
for DAF-2 and as low as 3 nmol/l in the more recently
developed derivative 4-amino-5-methylamino-2',7'-difluores-
ccein (32). Of note, DAF is probably not nitrosated by NO itself,
but rather by dinitrogen trioxide (N₂O₃), the product of NO
autoxidation. Moreover, the fluorescence yield is affected by
the stoichiometry between O₂⁻ and NO due to peroxynitrite-
dependent nitrosylation of DAF (17). Hence, the potential
influence of oxidative stress should be addressed in DAF
bioassays, e.g., by the use of antioxidants or superoxide dis-
mutase.

DAFs have been successfully applied to monitor NO pro-
duction in isolated, perfused rat lungs (3, 27, 38). After loading
of lung microvascular endothelial cells with the membrane-
permeable dye diacetate via a microcatheterization technique,
DAF imaging yields a dose-dependent fluorescence response to
an exogenous NO donor, S-nitroso-N-acetylpenicillamine (Fig.
2C). DAF imaging in intact lung microvessels presents a
powerful tool for in vivo studies of endothelial NO regulation,
as well as for the analysis of endothelial dysfunction, its
underlying mechanisms, and potential treatment options under
various pathophysiological conditions. Previously, this tech-
nique has allowed the unraveling of cellular signaling path-
ways underlying endothelial NO responses to mechanical stim-
uli, such as microvascular pressure (Fig. 2D) or shear stress (3,
38). Of note, fluorescent NO chelotropic traps were introduced
a few years ago for direct fluorescence detection of NO with
high sensitivity (49). Yet a broader application of this interest-
ing group of functional dyes has not yet been carried out.

Surfactant Secretion

A notable success has been the application of RFI for the
direct quantification of alveolar surfactant secretion. Tradition-
ally, lung surfactant secretion is determined indirectly through
quantification of surfactant phospholipid content in the bron-
choalveolar lavage, although bronchoalveolar lavage recovery
procedures may stimulate surfactant secretion (24). The RFI
approach uses the LysoTracker dyes (Molecular Probes),
which are fluorophore-linked weakly basic compounds that
become protonated and thereby sequestered in acidic or-
ganelles, such as surfactant-containing lamellar bodies (LBs).
In LysoTracker-loaded alveoli, LB fluorescence of type 2 cells
dominates over the weaker fluorescence of smaller lysosomal
inclusions and is conveniently detected as brightly lit fluores-
cent spots (Fig. 3). Loss of type 2 cell fluorescence denotes LB
exocytosis.

Using RFI, Ashino et al. (4) addressed cell-signaling mech-
anisms underlying lung inflation-induced surfactant secretion
(24). This mechanical effect is modeled in cultured type 2 cells
by cell-stretch protocols (13, 46, 60, 86) that identify Ca²⁺-cyt
increase as the major determinant of stretch-induced surfactant
secretion (46, 86). However, the studies by Ashino et al. (4)
reveal important differences in the stretch-induced Ca²⁺-cyt
response between cultured and intact alveolar cells. In cultured
type 2 cells, cell stretch induces external Ca²⁺ entry (46),
thereby markedly increasing mean Ca²⁺-cyt (86). However,
in the intact alveolus, the major Ca²⁺-cyt response to lung
inflation is a prolonged increase in Ca²⁺-cyt oscillations, but no
increase in mean Ca²⁺-cyt (4).

Gap junctional inhibitors blocked Ca²⁺-cyt oscillations in
type 2 cells, but not in type 1 cells, indicating that the
oscillations are transmitted by gap junctional communication
from type 1 to type 2 cells. This transmission is critical, since
inhibiting the oscillations inhibits LB exocytosis (4). The new
understanding gained from these studies is that type 1 cells act
as alveolar strain sensors. Thus, during inflation, type 1 cells
respond to alveolar stretch by transmitting Ca²⁺-cyt oscilla-
tions to type 2 cells, causing surfactant secretion.

NEW DIRECTIONS

Photolytic Uncaging

This method enables optically induced intracellular Ca²⁺
release (15), avoiding nonspecific effects of soluble ligands or
mechanical stimuli. For photolytic uncaging of Ca²⁺-cells, are
loaded with membrane-permeable, nitrophenyl (NP)-EGTA
AM that deesterifies intracellularly to NP-EGTA. Because of
its high Ca²⁺-affinity (Kₐ 80 nM), NP-EGTA acts as a “cage,”
sequestering free Ca²⁺ (16). Photo-excitation with high-inten-
sity UV light (320 nm) dissociates NP-EGTA into iminodiace-
tic products of low-Ca²⁺-affinity (Kₐ 3 mM), thereby releasing

Fig. 3. Real-time confocal microscopy of pulmonary
alveoli. Left: image shows alveoli (ALV) containing
type 2 cells loaded with lamellar body localizing dye,
LysoTracker Red (arrows). Note density of type 2 cells
is 1–2/alcove. Right: the region enclosed by the
dotted circle in the left image is shown at higher
magnification. Red LysoTracker Red fluorescence (ar-
rows) denotes lamellar body content and distribution.
the caged Ca\textsuperscript{2+} and increasing Ca\textsuperscript{2+} cyt. Uniquely, photolytic uncaging affords Ca\textsuperscript{2+} release in specific photo-targeted cells.

The application of photolytic release of caged Ca\textsuperscript{2+} in conjunction with RFI has enabled the detection of cell-cell communication in alveoli and lung capillaries. Ichimura et al. (26) loaded the Ca\textsuperscript{2+} cage in a cluster of alveoli (acinus) and determined Ca\textsuperscript{2+} cyt and surfactant secretion rates by the approaches reviewed above. Photolytic Ca\textsuperscript{2+} uncaging increased alveolar Ca\textsuperscript{2+} cyt and induced surfactant secretion, not only in the photo-targeted alveolus, but also in neighboring alveoli, indicating that Ca\textsuperscript{2+} is intercommunicated between adjacent alveoli. Pretreating the alveoli with the connexin 43 (Cx43)-inhibiting peptides blocked these conducted responses.

In the experiments by Parthasarathi et al. (58), in which NP-EGTA was loaded in the lung microvascular bed, photolytic uncaging revealed conduction of the evoked Ca\textsuperscript{2+} signal from septal capillaries to distances of \(-150\) \(\mu\)m from the uncaging site. Ca\textsuperscript{2+} increases induced in the alveolar capillary activated P-selectin expression in venules. As in the alveolar experiments, Cx43 peptides blocked capillary Ca\textsuperscript{2+} conduction. In addition, no conduction occurred in Cx43-deficient mice. These findings attest to a novel role for Cx43-mediated gap junctions as conduits for the spread of proinflammatory signals in the lung capillary bed. Taking together these uncaging studies in alveoli and capillaries, it appears that gap-junction-mediated Ca\textsuperscript{2+} conduction plays an important role in establishing defensive cell signaling in the lung.

**Optical-Sectioning Microscopy**

Confocal or multiphoton microscopy of optical sections eliminates blurring due to out-of-focus fluorescence that is inherent in standard, wide-angle microscopy (principles reviewed in Refs. 12, 23). Safdar et al. (66) applied confocal microscopy to determine F-actin distribution in lung venular capillaries, as determined by the fluorescence of rhodamine-phalloidin. Optical sections taken along a vertical axis revealed consistently greater rhodamine fluorescence at branch points, especially around ostia of tributary vessels (Fig. 4A, top). As tributaries progressively merged with the mainstream capillary, the intensity of actin fluorescence progressively decreased (Fig. 4A, middle and bottom). In general, actin fluorescence is higher at tributary branch points than at midsegmental locations (Fig. 4B), indicating that actin distribution is spatially nonuniform within a capillary segment.

In the same paper, Safdar et al. (66) determined endothelial cell geometry in venular capillaries in terms of fluo 4 fluorescence, which was bright in the nuclear region, but became attenuated toward the cell periphery (Fig. 4C). These peripheral sites were evidently cell junctions at which E-cadherin colocalized with actin. Interestingly, the capillaries failed to stain for VE-cadherin, the cadherin isoform detected in large-vessel endothelia. These findings indicate the usefulness of optical sectioning to access spatial data from three-dimensional anatomic structures.

In an interesting application of confocal microscopy, Carter et al. detected fluorescence emission from an optically fixed volume to quantify alveolar liquid flow (11). By filling the isolated, perfused mouse lung with buffer solution containing FITC-dextran, they achieved fluorescent labeling of the alveolar lumen. Images acquired at a series of \(z\)-positions were used to reconstruct the three-dimensional alveolar shape. To validate their measurements, they changed the osmolality of the vascular perfusate to induce water movement between the alveolar and perialveolar space. Perfusion with hyperosmolar buffer increased alveolar fluorescence compared with isosmolar conditions, indicating concentration of the fluorescent tracer, hence liquid removal from the alveolus. Accordingly, hyposmolar perfusate reduced alveolar fluorescence, consistent with liquid inflow into the alveolus.

The same group later extended this approach by filling the air space of mouse lung with fluorescent indicators of Na\textsuperscript{+} and Cl\textsuperscript{−} concentrations to determine unidirectional ion flux. The flux rates deduced by measurement of pleural surface fluorescence were validated by analysis of air space fluid samples. These findings have led to the understanding that cAMP-dependent Cl\textsuperscript{−} and Na\textsuperscript{+} transport occurs across the distal air space barrier into the alveolar lumen (29).

![Image](image_url)
Multiphoton microscopy provides a potentially promising approach for lung RFI studies. As different from conventional single-photon excitation, multiphoton fluorescence is excited by the combined energy of two or more low-energy (long wavelength) photons. This excitation is limited to the focal plane, since the probability of simultaneous absorption is negligible at other planes in the light path. This approach has several advantages over conventional confocal microscopy. First, the bulk of the fluorophore in the light path is unperturbed, although photobleaching might occur at the focal plane. Second, the incorporation of tunable lasers in the technology allows combined detection of UV and visible-light excited fluorophores (Fig. 5). Consequently, cells of different phenotypes are accurately localized in the alveolo-capillary region.

Third, the long wavelength excitation light used for multiphoton excitation provides a unique opportunity to detect lung connective tissue without the need for fluorescent staining. Light polarization in specific tissues causes second harmonic generation (SHG), which is a light signal at exactly one-half the excitation wavelength (44). SHG is typically generated by collagen in biological samples (89). Initial studies indicate that SHG can be applied for detection of collagen fibers on the lung surface and in alveolar septae (Fig. 5, bottom panels). This approach may find useful application in dynamic studies of lung fibrosis.

Fluorescence Energy Resonance Transfer

Fluorescence energy resonance transfer (FRET) occurs in the presence of two appropriate fluorophores (donor and acceptor) lying in close proximity, as for example when bound to different protein domains. Under these conditions, excitation of the donor results in partial energy transfer to the acceptor. If proximity of the donor to the acceptor changes, as for example by a conformational change in the protein, the amount of transferred energy changes, resulting in change in the ratio of the fluorescence emissions of the donor and acceptor. However, the ratio might also be determined by changes in protein dipole moment. To distinguish between these possibilities, donor fluorescence lifetimes are separately determined in the presence and absence of the acceptor. The true interfluorophore distance is determined by insertion of fluorescence lifetimes in the Förster equation (75). FRET pairs consisting of genetically encoded fluorescent proteins can be expressed in living cells and targeted to subcellular regions by specific promoters.

St. Croix et al. (73) applied this approach to transfect vascular endothelium of mouse lungs with cDNA encoding for a NO-sensitive metallothionein protein containing a cyan fluorescent protein/yellow fluorescent protein FRET pair. Transfection was confirmed after 48 h in isolated lungs, and the FRET response recorded by confocal microscopy indicated conformational changes of the metallothionein in response to a NO donor. Clearly, the application of FRET might enlarge the scope of RFI to address intracellular signaling at subcellular locations.

CONCLUDING REMARKS

We summarize here some of the main findings from real-time lung microscopy. In general, the microscopic approach to

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Fig. 5. Real-time multiphoton microscopy of the lung. Top: images of the alveolo-capillary region showing fluorescence of the UV-excited nuclear dye Hoechst 33342 (left), the Ca^{2+} dye fluo 4 (middle), and the corresponding overlay (right). Localization of nuclei allows identification of fluorescence profile at different locations of the capillary, as for example at branch points (arrow). Bottom: second harmonic generation (SHG) in the subpleural interstitium (left) and in alveolar septae (right, arrow). Note SHG of collagen fibers is considerably greater in the subpleural than in the alveolar interstitium.
in vivo and isolated, perfused lungs has evolved a novel understanding of lung proinflammatory signaling. The dynamic determinations with RFI portray the alveolo-capillary region as a field that is highly signaling sensitive. Rapid second-messenger responses incorporate different intracellular compartments and coordinate interactions among different cells. Importantly, for the first time, mitochondria are recognized as initiators of lung inflammation. New directions in lung RFI being developed are likely to reveal other presently unknown mechanisms. Wider application of real-time lung microscopy might establish a novel understanding of cell signaling underlying different processes of lung disease.

APPENDIX: NOTES ON METHODS

Fluorescent Dyes

RFI determinations have been obtained using cell-permeable fluorescent indicators. For example, the Ca$$^{2+}$$-sensitive dye fura 2 is membrane permeable in the AM form. In the cytosol, hydrolases cleave the AM moiety, leaving the dye membrane impermeable, hence cell located. However, dyes may be extruded from the cytosol by plasma membrane transporters, an effect best evident for the ROS-detecting dye DCF. To ensure steady cellular delivery of intracellular DCF, infusions of the precursor agent dichlorofluorescein diacetate are given throughout. For fura 2, dye content is indicated by the Ca$$^{2+}$$-insensitive fluorescence that is excited at wavelength of 360 nm.

Fluorescence indicators are either of the single- or dual-wavelength varieties. Dual-wavelength dyes that include fura 2 allow ratiometric imaging. Ratiometric quantification corrects for variability of cell volume and light absorption, which potentially introduces errors in quantitative interpretations of fluorescence emissions from single-wavelength dyes. For fura 2, in situ calibration allows direct interpretation of the imaged data in terms of the Ca$$^{2+}$$ cyt concentration. However, dyes such as fura 2 are excited at or near the UV range, excluding their application where use of UV is not indicated.

The advantage of single-wavelength dyes such as fluo 4 is that the dyes are excited in the range of visible light wavelengths and are, therefore, suitable for non-UV applications. Although the fluorescence emissions of these dyes do not provide a direct readout of the Ca$$^{2+}$$ cyt concentration, calibration against the concentration-calibrated fluorescence of a ratiometric dye such as fura 2 is possible by coloading both the single wavelength and the ratiometric dyes in the same cell (26). Alternatively, the cell volume problem associated with single-wavelength dyes may be suitably addressed by fluorescence determinations by optical sectioning microscopy.

Dye Loading

For lung microvascular RFI, one approach is to inject the dyes through a venous microcatheter wedged in the microvascular bed. Such microcatheter injections infuse a delimited microvascular region, protecting the bulk of the lung vasculature from dye exposure, hence reducing nonspecific fluorescence (background). While targeting capillary fluorescence, nonspecific fluorescence from extravascular cells may be eliminated by maintaining absorptive conditions in the vasculature during dye loading. Absorptive conditions are established by maintaining low vascular pressures and by controlling the solution colloid osmotic pressure of the dye at physiological levels.

For alveolar RFI, dyes are microinfused to fill seven to eight alveoli through a glass micropipette introduced in a single alveolus. Air withdrawal through the micropipette confirms correct micropipette tip placement in the alveolar lumen. Since alveolar microinfusions are rapidly (~2–4 s) removed (84), presumably by alveolar surface-active forces, dye loading requires prolonged microinfusions (20–30 min). Cells reseal spontaneously after micropipette removal (79); hence alveolar dye leakage does not occur from the micropuncture site. Faulty micropuncture that might tear the alveolar wall causes interstitial dye loading, the diffuse fluorescence of which is distinct from the discrete fluorescence of dye-loaded alveolar cells.

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